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**14. ABSTRACT**

The purpose of this study is to define correlations between indices of brain dysfunction, such as functional MRI (fMRI) and neuropsychological testing abnormalities, with oxidative phosphorylation (OXPHOS) defects in children with autistic spectrum disorders (ASD). This study is an essential step in identifying such a phenotypic subtype, being able to perform large-scale epidemiological studies using more widely available measures, and ultimately being able to implement clinical trials for new pharmaceutical agents emerging for treatment of the OXPHOS defects which could significantly improve the functioning of children with ASD with this defect. In the current year, we have begun analyzing all prior laboratory data and enrolling qualified subjects into the study. The patients are being stratified into appropriate groups for the fMRI analyses. Additionally, control ranges have been established for fibroblast high resolution respirometry.

**15. SUBJECT TERMS**
Autism, functional MRI, mitochondria, mitochondrial disease

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## Introduction

Although the precise frequency of mitochondrial defects in autism are not known, it is hypothesized that significant numbers of individuals with autism and autistic spectrum disorders (ASD) harbor oxidative phosphorylation (OXPHOS)defects important to ASD disease pathogenesis and/or functioning. These OXPHOS defects are identifiable in muscle, fibroblasts and EBV transformed lymphocytes. Pathogenic mutations in OXPHOS genes are predicted to be observed in patients with ASD at higher rates than in the general population. We propose that these OXPHOS defects correlate with indices of brain dysfunction such as functional MRI (fMRI) and neuropsychological testing abnormalities and define a specific subtype of children with ASD. This study is an essential step in identifying such a phenotypic subtype, being able to perform large-scale epidemiological studies using more widely available measures, and ultimately being able to implement clinical trials for new pharmaceutical agents emerging for treatment of the OXPHOS defects which could significantly improve the functioning of children with ASD with this defect.

## Body

**Brief background:**

Mitochondria are cytoplasmic structures with an inner and outer membrane separated by an intermembrane space. Oxidative phosphorylation (OXPHOS) is critical to cellular function as the primary source for energy (ATP) in most cell types, the control point for cellular redox, and as a control point for essential metabolic and signaling pathways that range from the synthesis of pyrimidines for the regulation of apoptosis. Substrates for ATP generation are derived primarily from glycolysis and fatty acid oxidation.

OXPHOS uses about 95% of the oxygen delivered to tissues, producing most of the ATP required by cells. Expression of genes involved in the OXPHOS pathway and the assembly of the five OXPHOS enzyme complexes Complex I (CI), Complex II (CII), Complex III (CIII, CIV and CV) within the inner mitochondrial membrane is a highly ordered and coordinated process directed by 37 genes in the mitochondrial DNA (mtDNA) and as many as 1,500 genes in the nuclear DNA (nDNA)[1,2].

Over 50 pediatric and adult diseases are caused by mutations in a heterogeneous array of OXPHOS genes coded by either the nDNA or the mtDNA. Genetic defects producing mitochondrial dysfunction include: (1) inherited mutations in nDNA or mtDNA genes. (2) Sporadic mutations occurring during embryogenesis that are systemic or confined to specific tissues such as skeletal muscle. (3) Somatic mutations occurring through life due to aging, free radical damage, and exposure to environmental toxins or certain medications. Defects in OXPHOS have a broad array of cellular

consequences including abnormal cellular calcium ($Ca^{2+}$) regulation, impaired ATP generation, enhanced apoptosis, and increased free radical production [3-6]. In fibroblast cell lines harboring pathogenic mutations in CI genes, CI dysfunction causes depolarization of the mitochondrial membrane potential, resulting in a decreased supply of mitochondrial ATP to the Ca2+-ATPases that control intracellular $Ca^{2+}$ stores. $Ca^{2+}$ content of these stores is then reduced, particularly in the endoplasmic reticulum [7]. Defects in any of these functions can lead to disease.

Most energy used for neuronal activity is expended as a result of the postsynaptic neuronal depolarization and to a lesser extent the action potentials generated [8]. OXPHOS uses approximately 95% of the oxygen delivered to tissues, thus making fMRI an important tool for non-invasive investigation of mitochondrial dysfunction. The energy cost arises from information transfer and its integration postsynaptically. Substrate delivery for energy metabolism is increased along with increased local blood flow in conjunction with to neurotransmitter action (local signaling). Reduced oxygen extraction as occurs with mitochondrial dysfunction leads to an increase in the ratio of oxy- to deoxyhemoglobin during neuronal activation.

Mitochondrial disease produces detectable fMRI abnormalities. For example, in Friedreich ataxia, a mitochondrial disease caused by abnormal iron incorporation into OXPHOS enzyme active centers [9], fMRI studies during motor tasks show cortical hypoactivity in a pattern consistent with the mitochondrial dysfunction [10]. fMRI abnormalities are well described in ASD and correlate with clinical features. Cortical hypoactivity in ASD includes fusiform gyrus which is associated with poor facial recognition [11] and anterior cingulate cortex which is related to inflexible and repetitive behavior [12]. Although the neuroanatomical substrates of the ASD phenotype are characterized, studies correlating fMRI findings with biochemical or molecular defects are lacking.

**Task 1: (Specific Aim I, human subjects, Months 1-30) Assessment of Neuropsychological Functioning. In order to make appropriate statistical analyses among neuropsychological data, fMRI data and laboratory data, the data analysis will extend to the end of the study (month 36). Appropriate numbers of patients are available for study.**

In order to complete this task, detailed clinical characterization and correlation with biochemical data on each patient must be performed. We have identified patients who are candidates for study and are in the process of clinically evaluating them for functional MRI study.

We are completing assessment of patients and their data for recruitment and for proper stratification of the patients for

fMRI and neuropsychological testing: ASD with mitochondrial defects and ASD without mitochondrial defects. This is a complex process due to the complexity of OXPHOS data. Data from 66 patient data is correlated in Table 1, demonstrating the stratification process. We are proceeding with completing evaluations in appropriate subjects.

Of these initial 66 patients surveyed, 35 are candidates for study in the mitochondrial dysfunction group and 31 are candidates in the NO mitochondrial dysfunction group.

**Data for Stratification:** The ASD patients are evaluated for stratification into two groups according to complex biochemical criteria. The data are divided into the following sections:

1) Biochemical data on muscle. Extensive testing was performed on patient muscle in order to assess mitochondrial function. The categories of testing are as follows:

a. OXPHOS enzymology. Specific activities of Complexes I, II, III, and IV are testing in isolated muscle mitochondria. The specific activity measurements are compared to the control ranges.

b. High Resolution Respirometry which assesses many aspects of mitochondrial function in living cells including coupling and proton leak across the inner mitochondrial membrane.

i. High resolution respirometry is a highly sensitive approach for assessing Complex I-V function as well as coupling of mitochondrial proton translocation to ATP generation (Complex V). Testing is performed using the Oxygraph 2K (Oroboros, Austria) at 37°C. Instrumental and chemical oxygen background fluxes are determined with each assay and corrected over the entire experimental oxygen range. An automatic injector is used to investigate the effects of the uncoupler (FCCP) over a wide range of concentrations (0.5 µM increments). This approach allows accurate assessment of the coupling of ATP synthesis to proton translocation in patient samples. Cellular respiration in the uncoupled state and uncoupled state are compared for assessment of metabolic flux control by the phosphorylation system (Complex V) over the electron transport capacity (Complexes I-IV).

c. Muscle mitochondrial CoQ10 level which screens for hereditary defects in CoQ10 synthesis.

d. Quantitative Western blot of OXPHOS proteins.

i. Western blot is used to assess levels of individual OXPHOS subunits. This technique is sensitive to a variety of defects that alter mitochondrial function and highly reproducible. This approach assesses representative subunits of each OXPHOS enzyme complex using a cocktail of subunit specific monoclonal antibodies (Mitosciences, Eugene, Oregon): (1) Complex

I ND6 subunit (mtDNA coded), (2) Complex II 30 kDa subunit (nuclear DNA coded), (3) Complex III core protein 2 (nuclear DNA coded), (4) Complex IV subunit II (mtDNA coded), (5) Complex V alpha subunit (nuclear DNA coded). The subunits are resolved by SDS-PAGE, detected by chemiluminescence. Signal is normalized to GAPDH (a constitutively expressed enzyme). Normal control tissue and tissue from a patient harboring a pathogenic mtDNA mutation (MELAS, 3243A>G) are included on each gel.

e. Muscle OXPHOS supercomplex formation and monomeric enzyme assembly. Alterations in supercomplex formation are common to numerous categories of mitochondrial disease. This aspect of OXPHOS is assessed carefully in the ASD population and is essential for proper stratification. This feature of the ASD patients is assessed by blue native and clear native gel analysis.

i. Blue Native Gels (BNG) and Clear Native Gels (CNG): BNG and CNG analyses are sensitive techniques used to assess each OXPHOS enzyme (Complexes I-V) in fibroblasts and EBV transformed lymphoblasts. BNG and CNG also assess supercomplex formation. This type of analysis has been performed with muscle, platelets and fibroblasts from patients with suspected mitochondrial diseases[13-18]. For BNG, multisubunit OXPHOS enzymes bind a charged dye (Coomassie brilliant blue) which allows electrophoretic separation in the first dimension by the size of the complex. Complexes I-V with masses ranging from 950K to 200K are well resolved in the first dimension. The individual complexes can then be dissociated and electrophoresed into the second dimension allowing inspection of the integrity of all OXPHOS enzyme subunits. CNG allows assessment of assembly of the OXPHOS enzyme complexes as well as in gel enzyme activity measurements to assess OXPHOS enzyme function.

**Table 1: Distribution of OXPHOS ABNORMALITIES in ASD patients. The following data is used for stratification into the two groups discussed above.**

| Biochemical OXPHOS Test | Results Summary (% ABNORMAL) |
|---|---|
| OXPHOS Enzymology(Complexes I-IV) | 63.6% (42/66) |
| High Resolution respirometry (live cells) | 28.6%(10/35) |
| Muscle CoQ10 level | 2.7% (1/36) |
| OXPHOS subunit Western blot | 34.1%(14/41) |
| Blue Native and Clear Native Gel Testing | 1. OXPHOS Supercomplex formation 50% (19/38)<br>2. Monomeric Enzyme Assembly: 52.6% (20/38)<br>3. Clear Native Gel of Intact OXPHOS enzymes: Abnormal in-gel enzyme activity was observed in 36.8% (14/38) |

In addition to the above data that is used for stratification, we reviewed and summarized metabolic data available from cerebrospinal fluid, blood, and urine testing. Results of key metabolic parameters important in OXPHOS diseases are summarized in Table 2 below. The data below provides important insights into the ASD patients. Cerebral folate deficiency (CFD) is characterized by decreased concentrations of 5-MTHF in the CSF in the context of normal systemic folate metabolism. CFD can occur in up to 40% of all patients with mitochondrial disease [19]. The mechanisms involved have yet to be clarified but oxidation of these labile compounds or decreased transport into the CNS due to reduced ATP levels have been suggested as possible mechanisms [20].

In total, we have obtained ten consents for the study. Of these, two have ADI tests in progress. We expect to enroll a majority of the participants in years two and three of the study.

**Table 2: Summary of metabolic parameters in ASD patients. The following data is used for stratification into the two groups discussed above.**

| Cerebroaspinal(CSF) Fluid Metabolite Tested | Results Summary (% ABNORMAL) |
|---|---|
| CSF lactate | 3.8% (2/52) |
| CSF Pyruvate | 0% (0/52) |
| CSF 5-Methyltetrahydro-folate | 14% (8/57) |

| Blood Metabolite Tested | Results Summary (% ABNORMAL) |
|---|---|
| Blood lactate | 33% (18/54) |
| Blood Pyruvate | 30% (16/53) |

| Urine Metabolite Tested | Results Summary (% ABNORMAL) |
|---|---|
| Urine Amino Acids | 11% (6/53) |
| Urine Organic Acids (abnormal lactate, Kreb cycle intermediates, etc) | 10% (5/50) |

**Task 2: (Specific Aim II, human subjects, Months 1-30) Neuroimaging: Due to the detailed and time consuming nature of fMRI data analysis, data analysis will begin during the fMRI acquisition period. In order to make appropriate statistical analyses among neuropsychological data, fMRI data and laboratory data, the data analysis will extend to the end of the study (month 36).**

Data analysis continues throughout the study. No data is ready for presentation at this juncture. We are completing appropriate stratification of patients as discussed in Task 1 above.

**Task 3: (Specific Aim III, Banked samples that include muscle, fibroblasts, EBV transformed lymphocytes are used for study) Months 1-30. Although data analysis will be proceeding during the study, the last 6 months are reserved for assessing the data obtained from all specific aims (tasks).**

We have made significant progress on this specific aim (task). Analysis is complex requiring comparison of patient with autism to various categories of normal control cell lines and disease controls.

According to the statement of work, months 1-12 are used used to establish the reference intervals for each analysis. Each of the techniques will be validated by comparison with patient cell

lines (fibroblast and EBV transformed lymphoblasts) who harbor known mitochondrial DNA mutations or nuclear OXPHOS gene mutations. A variety of classes of mutations will be used to understand how the various mitochondrial disease mechanisms affect the results of each test. The next 12 months are used to analyze autism cell lines.

**Fibroblast Assessment:** Specific Aim III proposes that fibroblasts will be used for assessment of mitochondrial dysfunction.(Table 1) The grant proposes assessment of:

1. High Resolution Respirometry
    a. Control ranges have been established for appropriate testing parameter. The reference interval data is given below.
        i. Uncoupling Ratio
        ii. Net Routine Flux Control Ratio
        iii. Respiratory Control Ratio
        iv. Leak Flux Control Ratio

**Table 3: Control ranges for fibroblast high resolution respirometry.**

| | Range | 5% | 25% | Mean | 75% | 95% |
|---|---|---|---|---|---|---|
| Uncoupling Ratio | 2.30-3.13 | 2.28 | 2.52 | 2.70 | 2.87 | 3.12 |
| Net Routine Flux Control Ratio | 0.320-0.435 | 0.316 | 0.350 | 0.373 | 0.397 | 0.430 |
| Respiratory Control Ratio | 7.60-14.88 | 7.10 | 9.28 | 10.79 | 12.30 | 14.47 |
| Leak Flux Control Ratio | 0.067-0.131 | 0.064 | 0.083 | 0.097 | 0.110 | 0.129 |
| Phosphorylation Respiratory Control Ratio | 0.221-0.355 | 0.218 | 0.253 | 0.277 | 0.301 | 0.335 |

    b. So far 25 patients with ASD have been analyzed by high resolution respirometry. The data is compared to the control reference intervals. 14 of the 25 ASD tested are abnormal (56%). This is highly significant data which suggests that oxidative phosphorylation (OXPHOS) defects in patients with ASD may be able to be screened using fibroblasts rather than more invasive techniques like muscle biopsies. As we proceed with the grant and complete the analyses in #2 and #3 below, we will have a more complete assessment of how diagnosis of OXPHOS defects in fibroblasts compares with muscle biopsy techniques.
2. Quantitative Western blot of OXPHOS proteins
    a. Control ranges are being established.
    b. Once control ranges are established, they will be used for assessment of ASD patients.
    c. In addition the data from the fibroblasts will be correlated with muscle data that is available on the ASD patients
3. Blue Native and Clear Native Gels

a. Control ranges are being established.

b. Once control ranges are established, they will be used for assessment of ASD patients.

c. In addition the data from the fibroblasts will be correlated with muscle data that is available on the ASD patients

**Task 4: (Specific Aim 4, Banked DNA Analysis) Months 12-30**
According to this specific aim (Task), this work will begin during year two.

## Key Research Accomplishments

1. The patient population is the most comprehensively assessed group in the country for mitochondrial defects.
2. Fibroblast high resolution respirometry may be an important method of screening for OXPHOS defects in ASD.
   a. Control ranges in fibroblasts have been carefully established.
3. Abnormalities in supercomplex formation are highly sensitive for diagnosis of mitochondrial diseases.
4. 14% (8/57) ASD patients harbor cerebral folate defects. Cerebral folate defects are prevalent in patients with mitochondrial diseases. This is an important observation in these patients since cerebral folate defects (decreased CSF 5-methyltetrahydrofolate levels) can be treated.
5. The patient are being stratified for functional MRI and neuropsychological study according to rigorous criteria outlined in the tables above for identifying patients with OXPHOS disease (also called mitochondrial disease).

## Reportable Outcomes

None at this juncture in the study.

## Conclusions

At this early phase in the study, we have significant data for dividing patients into appropriate study groups. Recruitment and screening of patients for functional MRI studies is under way. Laboratory investigations are proceeding according to the SOW schedule.

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## Appendices

None at this juncture in the study.